Gene Expression Patterns of the Amphibian HPT-Axis in Normal Development and After Exposure to the Modulators Methimazole and Perchlorate

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Abstract

In response to the Endocrine Disruptor Screening and Testing Program Advisory Committee (EDSTAC) recommendations, the US EPA has been developing a screening test capable of detecting the effects of Endocrine Disrupting Chemicals (EDCs) on the hypothalamus-pituitary-thyroid (HPT) axis in *Xenopus laevis*. The screening test compares morphometric (altered development), biochemical (thyroid hormone concentrations), and gene and protein expression measurements. As part of this, we determined expression patterns of key genes from Nieuwkoop and Faber stages 51 to 64 to establish a baseline to which potential changes caused by modulators of normal HPT functioning could be assessed. In the pituitary we measured thyroid stimulating hormone (TSH) and deiodinases type II and III (D2 and D3). In the thyroid gland we measured thyroid transcription factor I (TTF I), thyroid peroxidase (TPO), thyroglobulin (TG), and sodium-iodide symporter (NIS). We then measured the expression of these genes after exposure to the HPT-modulators methimazole and perchlorate. Measurements were made by quantitative real-time RT-PCR (Q-PCR) on total RNA extracts. The results are expressed on a per gland basis and after normalization to the amount of total RNA present. Significant patterns of stage-specific gene expression were observed in the developmental studies. The modulators caused increases in TSH expression in the pituitary, as expected, although the magnitude was not large. D2 expression was generally similar to controls, but may show some early transient increases. D3 expression was low and variable, as would be predicted based on the developmental expression at these stages. The modulators caused large changes in expression of the thyroid genes, but the changes were generally similar to controls when normalized. This suggests the changes were not necessarily caused by specific induction of the genes, but reflective of the overall increase in size of the thyroid. However, the increased expression of NIS was still evident after normalization, indicating a specific induction of this gene.

Methods

•*Xenopus Laevis* tadpoles were from in-house cultures. Staging was according to Nieuwkoop and Faber. Exposures began with stage 54 organisms under flow-through conditions at 100 ppm for methimazole and 4 ppm for perchlorate.

•RNA extraction was done with Qiagen's micro RNeasy kits and concentrations were determined using a Nanodrop spectrophotometer. RNA quality was assessed with Agilent's Bioanalyzer using the picochip format.

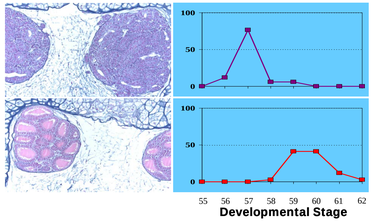
•Sequence information was obtained from NCBI for TSH, D2, D3, TTF-1, and NIS. Sequence information for TG was obtained from TIGR. Degenerate/consensus primers and PCR were used to amplify a segment of TPO which was verified by sequencing.

•Q-PCRs were performed on Applied Biosystem's 5700 and 7500 instruments, all primer and probes were designed with Primer Express. Measurements were made on 4 to 6 individuals per treatment or stage of development. Statistical significance ($p = 0.05$) is indicated based on the result of t-tests between control and treatment at each time. For some, the data were log transformed to account for differences in variability between groups.

Biological Effects

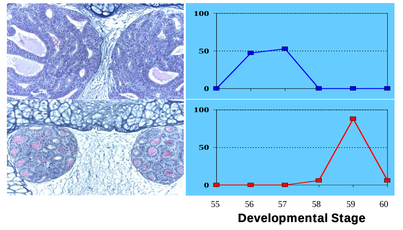
Effect of 14 Day Perchlorate Exposure on stage 54 *X. Laevis*

Perchlorate µg/l	Final Stage							
	55	56	57	58	59	60	61	62
Control				1	14	14	4	1
16				2	16	13	2	1
63			1	2	18	12	1	
250			8	5	17	4		
1,000		4	26	2	2			
4,000	3	26	5					

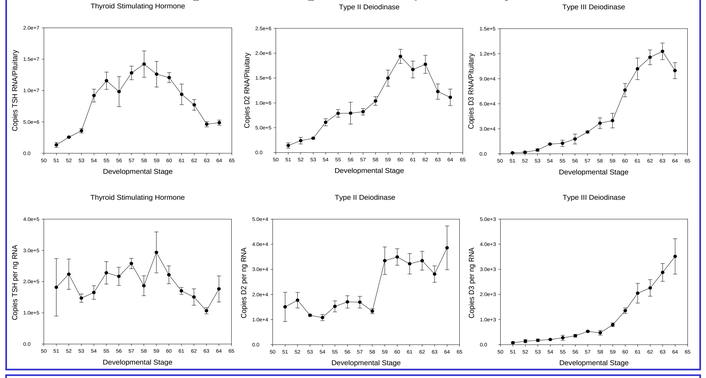


Effect of 14 Day Methimazole Exposure on Stage 54 *X. laevis*

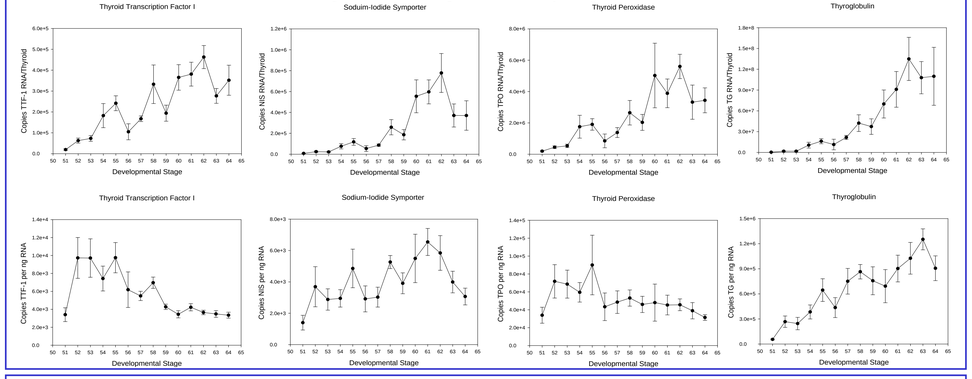
Methimazole mg/L	Final Stage					
	55	56	57	58	59	60
Control				2	30	2
6.25			5	6	19	4
12.5			3	15	13	2
25		16	18			
50		26	6			
100	4	30				



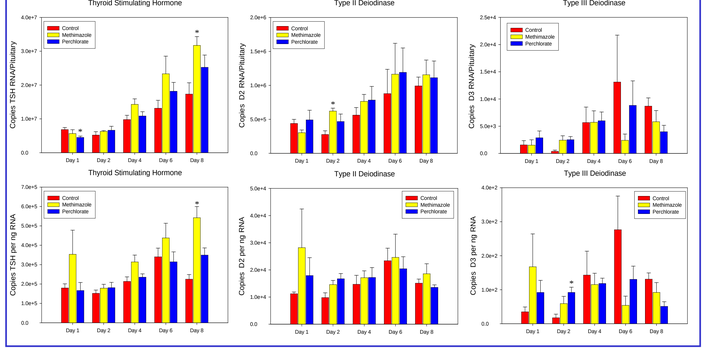
Developmental Expression of Pituitary Genes



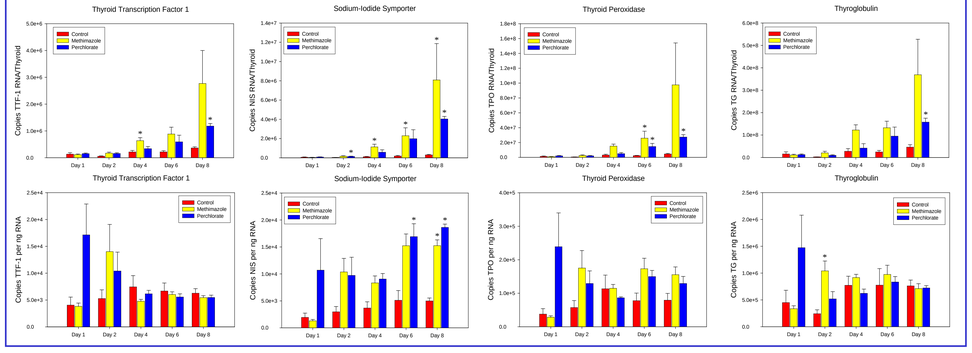
Developmental Expression of Thyroid Genes



Pituitary Gene Expression after Exposure to Modulators



Thyroid Gene Expression after Exposure to Modulators



Conclusions

•Developmental expression of pituitary genes on a per gland basis shows a broad TSH peak at stages 57-59. This is earlier than either D2 (stages 60-62) or D3 (stages 62-63). After normalization with RNA, the TSH peak is not as distinct, but the D2 pattern indicates a steep change between stages 58 and 59. Expression of D3 increases steadily throughout development.

•The effects of the modulators on pituitary gene expression were less than anticipated, especially for TSH where it was thought that the observed thyroid hypertrophy is due to increased TSH hormone levels. However, there were increases in TSH due to both modulators, and it is possible that a small change in gene expression results in greater relative changes at the protein level.

•Expression of all measured thyroid genes increased in a similar manner throughout development. They appear to be reflecting the increase in the size of the gland itself. After normalization, TTF-1 and TPO tend to show decreased relative expression with advancing development, while NIS and TG show increasing relative expression.

•Both methimazole and perchlorate caused large increases in expression of thyroid genes on a glandular basis. After normalization, these increases were much less striking, indicating that control of expression was not specifically directed at these genes, but was more reflective of increased thyroid size. However, NIS continued to show increased expression after normalization, indicating a specific up-regulation of this gene. This response seems reasonable as increased TG will increase demands for more iodine.

• Direct comparison of modulator effect is challenging due to several factors, including the induced developmental delay. Comparison to stage-matched controls may be more appropriate. This is further complicated by fluctuations in the expression of genes in normal development (e.g. TTF-1). Also, there are indications that some transient changes may be occurring (e.g. TTF-1) which is followed by a return to control levels. There also seems to be increased variability after exposure, indicating a dynamic response.

Future Directions

•Currently analyzing samples with Affymetrix' *X. laevis* gene arrays

•Develop ELISAs/RIAs to measure specific proteins, e.g. TSH

•Proteomic analysis by 2-D LC/MS and 2-D PAGE is in progress

•Examination of expression patterns in other tissues, e.g. limb

•Development and transfer of methods to *Xenopus tropicalis*

•Correlation of gene expression with thyroid hormone measurements